

ORIGINAL ARTICLE

Detection of Respiratory Viruses among Chronic Rhinosinusitis Patients Attending Zagazig University Hospitals

¹Enas A. Tantawy*, ¹Somaya M. ElSheikh, ¹Ghada A. Mokhtar, ²Mohammad A. Mohammad

¹Microbiology & Immunology Department, Faculty of Medicine, Zagazig University

²Otorhinolaryngology Department, Faculty of Medicine, Zagazig University

ABSTRACT

Key words:

Chronic rhinosinusitis, respiratory viruses, multiplex nested PCR

***Corresponding Author:**

Enas A. Tantawy,
Microbiology and
Immunology Department,
Faculty of Medicine, Zagazig
University, Egypt.
Tel: 01000837372.
enasatantawy@gmail.com

Background: Chronic Rhinosinusitis (CRS) is characterized by inflammation of nose and paranasal cavities. High prevalence of respiratory viruses was detected in CRS patients. **Objectives:** To investigate prevalence of respiratory viruses in nasal mucosa of CRS patients and their association with allergic conditions. **Methodology:** The study included 30 CRS patients and 30 controls. Nasal wash samples were obtained from participants and nasopharyngeal swabs from 10 patients and controls, viral RNA extraction and multiplex nested PCR were performed to detect respiratory viruses. **Results:** Respiratory viruses were present in 53.3% of patients and 16.6% of controls ($P= 0.003$). Respiratory viruses were detected in 57.9% of allergic rhinitis and 50% of asthma patients. Comparing between sampling techniques showed that nasopharyngeal swabbing detected respiratory viruses in 70% of cases and 50% of controls, while nasal wash samples revealed viruses in 30% of patients and 10% of controls. Rhinovirus (RV) was the most commonly detected virus. **Conclusions:** Respiratory viruses are prevalent with CRS. The co-presence of CRS with AR and/or asthma suggests their relationship to disease progression. Multiplex nested PCR facilitated rapid and sensitive virus detection.

INTRODUCTION

Chronic Rhinosinusitis (CRS) is a highly prevalent disease characterized by inflammation of the nose and paranasal cavities ¹. It is defined by at least 3 months of cardinal sinonasal complaints as nasal obstruction, nasal discharge, facial pain or olfactory dysfunction along with visible evidence of inflammation on either physical examination or diagnostic imaging ².

CRS is usually divided into three subtypes; CRSsNP (Chronic Rhinosinusitis without Nasal Polyp), CRSwNP (Chronic Rhinosinusitis with Nasal Polyp), and AFRS (Allergic Fungal Rhinosinusitis) ³. CRSsNP was thought to be a result of an inadequately treated or unresolved bacterial infection, while CRSwNP was regarded as a noninfectious disorder linked to atopy ⁴. The cytokine profile of CRSsNP is mainly Th1 dominant, patients with CRSwNP have a predominantly Th2-biased eosinophilic inflammation, while, AFRS is accompanied by allergic mucin containing degranulated eosinophils and fungal hyphae ⁵. CRS may also occur as a local manifestation of systemic diseases, autoimmune disorders or immunodeficiencies ⁶.

The airway is a continuous structure extending from nasal vestibule to alveoli of the lung and its mucosal surface is constantly exposed to the outside world. Dysregulation of its function is implicated in the pathogenesis of allergic rhinitis (AR), CRS and asthma, so the common coexistence of these diseases and the overlap in their immunopathology suggests they are

strongly related ⁷. Allergic Rhinitis often predisposes to CRS evidenced by their co-presence in high percentage of patients, and also by the presence of elevated total IgE levels in 97% of patients with AR and chronic sinusitis ⁸. Moreover, non allergic asthma was found as a common association with CRS and the extent of sinonasal disease correlates with asthma severity ⁷.

Respiratory viral infections have important role in acute rhinosinusitis as they induce impairment of tight junctions in nasal epithelial cells and facilitate invasion of bacteria into nasal mucosa ^{9,10}. Symptoms of CRS commonly develop in patients after complaining of viral infections with obstruction of the sinus ostia and production of inflammatory mediators ¹¹. Moreover, the high prevalence of respiratory viruses, particularly the rhinovirus (RV), detected in nasal samples from CRS patients support the association between respiratory viral infection and CRS ¹².

The aim of this work is to investigate the prevalence and types of respiratory viruses in nasal mucosa of CRS patients in Zagazig University Hospitals and to explore the possible relationship between the presence of those respiratory viruses and associated allergic conditions.

METHODOLOGY

Study subjects and design:

This case-control study was conducted over the period from March 2015 to March 2016 in Microbiology & Immunology and Oto-Rhino-

Laryngology Departments, Faculty of Medicine, Zagazig University.

The study included 60 participants in two groups; a patients' group of 30 CRS patients (9 males and 21 females) with ages ranging from 6-65 years recruited from the Oto-Rhino-Laryngology Outpatient Clinic and the Allergy Unit of Microbiology & Immunology Department. Together with a control group of 30 (9 males and 21 females) apparently healthy individuals with no history of CRS, matched with patients for age and sex.

Inclusion and exclusion criteria

The study included patients diagnosed with uncontrolled CRS after optimal medical treatment. They suffered from nasal obstruction, anterior and posterior rhinorrhea, hyposmia and mid face congestion. Patients did not have any viral upper respiratory infections during the previous 4 weeks¹³. Associated allergic diseases were diagnosed by thorough history taking, clinical examination, spirometry (for asthma) and intradermal skin test at Allergy Unit¹⁴. Exclusion criteria included; Fungal sinusitis, current immunotherapy, aspirin exacerbated respiratory disease, cystic fibrosis, treatment with local or oral steroids within the previous 4 weeks^{11,13} or participant refusal.

Ethical considerations

All participants gave informed consents after explaining the nature as well as the purpose of this work. The study was approved by the Institutional Review Board (IRB) of Faculty of Medicine, Zagazig University.

Samples collection:

Two methods of sample collection were used in this study; the nasal wash method for all participants using sterile phosphate buffer saline solution (PBS) at room temperature introduced into each nostril using a needle-free syringe until 10 ml of lavage fluid were recovered¹³. The second method was using nasopharyngeal swabs (NPS) and oropharyngeal swabs (OPS), which were obtained from ten patients of the nasal wash group and their corresponding controls. A nasopharyngeal swab was performed using a flexible flocked swab with a nylon tip and plastic applicator (**Copan, Italy**) inserted into one of the nostrils until slight resistance was felt at the nasopharynx. The swab was then rotated two to three times, held in place for 5 seconds then withdrawn. Oropharyngeal swabs were used to collect nasopharyngeal secretions from the posterior pharyngeal wall using sterile cotton swabs emerged in sterile PBS, then transported in sterile epindorff tubes containing 2ml of sterile PBS after cutting-off its applicator. The NP and OPS swab samples were vortexed and centrifuged. The supernatant and cellular materials from samples were frozen and stored at -80°C until used for molecular techniques¹⁵.

Detection of respiratory viruses by multiplex nested Polymerase chain Reaction (PCR):

Viral RNA extraction:

Viral RNA extraction was performed using QIAamp Viral RNA Mini kit (**ROCHE, Germany**) according to the manufacturer's instructions.

Reverse transcription of extracted viral RNA:

Reverse transcription was performed to synthesize cDNA from the extracted viral RNA using The QuantiTect Reverse Transcription kit (**ROCHE, Germany**) according to manufacturer's instructions, using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction was performed at 42°C and then inactivated at 95°C .

Multiplex nested PCR amplification of cDNA:

Multiplex nested PCR was carried-out to detect different respiratory pathogens simultaneously, the test included influenza virus group A (FluA) (subtypes H1, H3, and H5), influenza virus group B (FluB), parainfluenza virus type 1 (PIV-1), PIV-2, PIV-3, PIV-4, human respiratory syncytial viruses (hRSV), human metapneumoviruses (hMPV), human coronaviruses (HCoV) (HCoV-229E, HCoV-OC43), human enteroviruses (hEVs), and human rhinoviruses (hRVs), reactions were all performed using the QIAGEN Multiplex PCR Kit (**ROCHE, Germany**) according to the manufacturer's instructions.

Two multiplex nested PCRs, were performed for each sample with two sets of primers¹⁶. The reactions were performed in 50 μl reaction mixtures. The first round mixture contained 25 μl of QIAGEN Multiplex PCR master mix, 2 μl template cDNA, 1 μl IC forward primer, 1 μl IC reverse primer, RNase free water and primers. The second reaction contained 25 μl of QIAGEN Multiplex PCR master mix, RNase free water, primers and 0.2 μl of the first round PCR products as template DNA, it was very unlikely that any of the unwanted PCR products contained binding sites for the new primers, thus ensuring that the amplicon from the second round of nested PCR would not have any undesired products. The amplified PCR products were visualized by agarose gel electrophoresis using agarose gel (2%).

Statistical analysis

Data were coded, checked, entered and analyzed using Statistical Package for the Social Sciences (SPSS) version 22 (SPSS Inc., Chicago, Illinois, USA). Results of the quantitative variables were expressed as means \pm standard deviation (SD). Student's "t" test was used to ascertain significance of differences between mean values of two continuous variables. Categorical variables were compared by chi-squared-test (X^2).

RESULTS

Characteristics of the studied subjects

This study was conducted on 30 CRS patients and 30 apparently healthy control subjects, whose demographic and clinical data are shown in table (1).

Patients and controls were matched for age and sex. The majority of CRS patients were females (70%), versus 30% male patients, with no statistically significant difference ($P=1.00$). Associated comorbidities were; 63.3% allergic rhinitis, 33.3% asthma, 6.6% urticaria, 3.3% conjunctivitis, 10% diabetes & 3.3% hypertension.

Table 1: Demographic and clinical data of patients and control subjects.

	Cases (No=30)		Control (No=30)		P-value
	Mean±SD		Mean±SD		
Age (years)	32.3±13		33.8±13.1		0.65
Sex	No.	(%)	No.	(%)	1.00
Male	9	30	9	30	
Female	21	70	21	70	
Comorbidities	No.	(%)	NA	NA	
Allergic rhinitis	19	63.3			
Asthma	10	33.3			
Urticaria	2	6.6			
Conjunctivitis	1	3.3			
Diabetes	3	10			
Hypertension	1	3.3			

NA: not applicable

On performing allergy intradermal skin tests, smoke was the most common allergen (66.6%) followed by pollens (53.3%), house dust mites (33.3%), hay dust (30%), mixed fungus (23.3%), cotton and wool (3.3% each).

Detection of respiratory viruses by multiplex nested PCR

Analysis of nasal wash samples by multiplex PCR revealed the presence of respiratory viruses in 53.3% of patients, and 16.6% of control samples, with a statistically significant difference ($P= 0.003$) (table 2). Respiratory viruses were detected in 57% of female and 44% of male patients, with no statistically significant difference ($P=0.52$).

Table 2: Respiratory viruses in nasal wash samples of the studied groups.

Respiratory Viruses	Nasal wash		Cases (No= 30)		Control (No= 30)		P-value
	No.	(%)	No.	(%)	No.	(%)	
Present (+ve)	16	53.3	5	16.7			0.003
Absent (-ve)	14	46.7	25	83.3			*

*significant

We investigated the relation between the presence of respiratory viruses and associated allergic conditions and we found respiratory viruses in 57.9% of CRS patients with allergic rhinitis and in 50% of CRS patients with asthma, yet with no statistically significant result ($P= 0.5$ & 0.7 , respectively) (table 3).

Table 3: Respiratory viruses in nasal wash samples of CRS patients with allergic rhinitis and bronchial asthma.

Respiratory viruses	Allergic rhinitis				p-value	Asthma				p-value
	Present (19)		Absent (11)			Present (10)		Absent (20)		
	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Present (+ve)	11	57.9	5	45.5	0.5	5	50	11	55	0.7
Absent (-ve)	8	42.1	6	54.5		5	50	9	45	

Rhinovirus (RV) was the most commonly detected respiratory virus in nasal wash samples of our patients (75%), followed by Influenza A virus, human coronavirus (HCoV-OC43), Enterovirus and Parainfluenza virus (PIV-1) (6.2%). Also, RV was the

most commonly found virus (60%) in the control group, followed by Influenza A virus (40%), while none of the other viruses were found in nasal wash samples from controls (figure1).

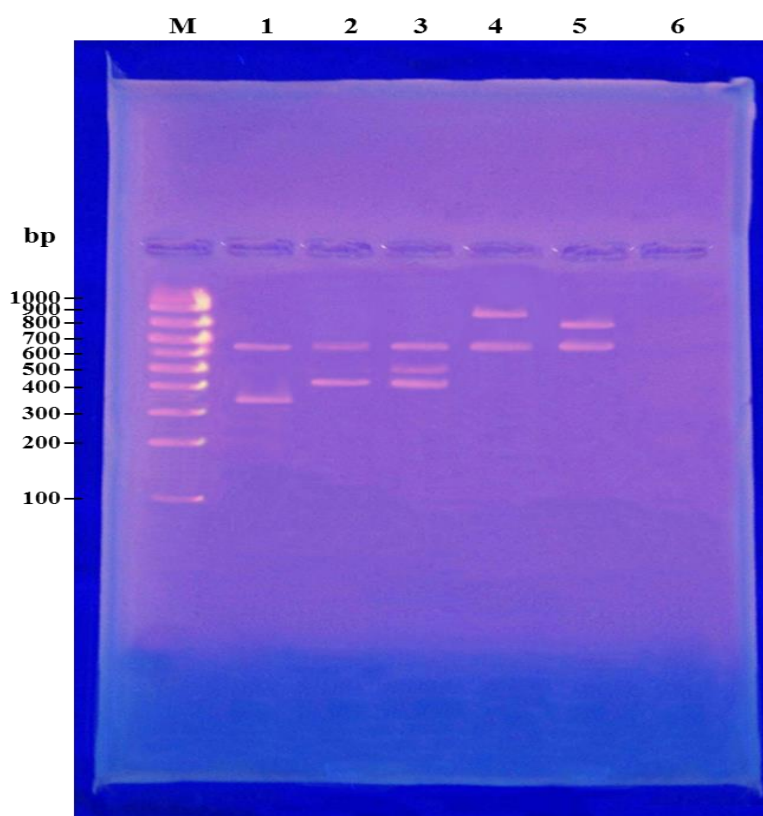


Fig. 1: Respiratory viruses in nasal wash samples of cases showing RV, enterovirus, influenza A virus, PIV-1, HCoV-OC43 in the first round of multiplex nested PCR.

The comparison between the results obtained from samples of the nasal wash and nasopharyngeal swabbing techniques performed on 10 patients and their corresponding control volunteers, showed that nasopharyngeal swab method detected respiratory

viruses in 70% of cases and 50% of controls, while nasal wash samples revealed viruses in only 30% of patients and 10% of controls, yet this result was statistically insignificant ($P=0.125$) (table 4).

Table 4: Detection of respiratory viruses by nasal wash compared to nasopharyngeal swabbing in the studied groups.

Nasopharyngeal swab \ Nasal Wash	Cases (No=10)				Controls (No=10)			
	Present (No=7;70%)		Absent		Present (No=5;50%)		Absent	
	No.	%	No.	%	No.	%	No.	%
Present (+)	3	30	0	0	1	10	0	0
Absent (-)	4	40	3	30	4	40	5	50
P-value	0.125				0.125			

The nasal wash technique detected only a single respiratory virus in 30% of patients and 10% of controls. While, nasopharyngeal swab detected a single

virus from 40% of both patients' and control samples, in addition to multiple virus detection from 30% of patients and 10% of control samples (table 5).

Table 5: Single and multiple respiratory viral detection by nasal wash compared to nasopharyngeal swabbing.

	Respiratory viruses			
	Cases (No =10)		Controls (No =10)	
	No.	(%)	No.	(%)
Nasal wash				
Single virus	3	30	1	10
Multiple viruses	0	0	0	0
Nasopharyngeal swab				
Single virus	4	40	4	40
Multiple viruses	3	30	1	10

Regarding the types of respiratory viruses in positive nasopharyngeal swab samples, only RV was detected in samples from 4 patients, while RV & PIV-1 were found in one patient, RV & Influenza A viruses in another patient, whereas the co-existence of RV, PIV-1 &

enterovirus occurred in one patient. Positive nasopharyngeal swab samples from control subjects showed that 3 samples had only one of RV, Influenza A, enterovirus or PIV-1 virus, and a co-infection was detected in one sample (figure 2).

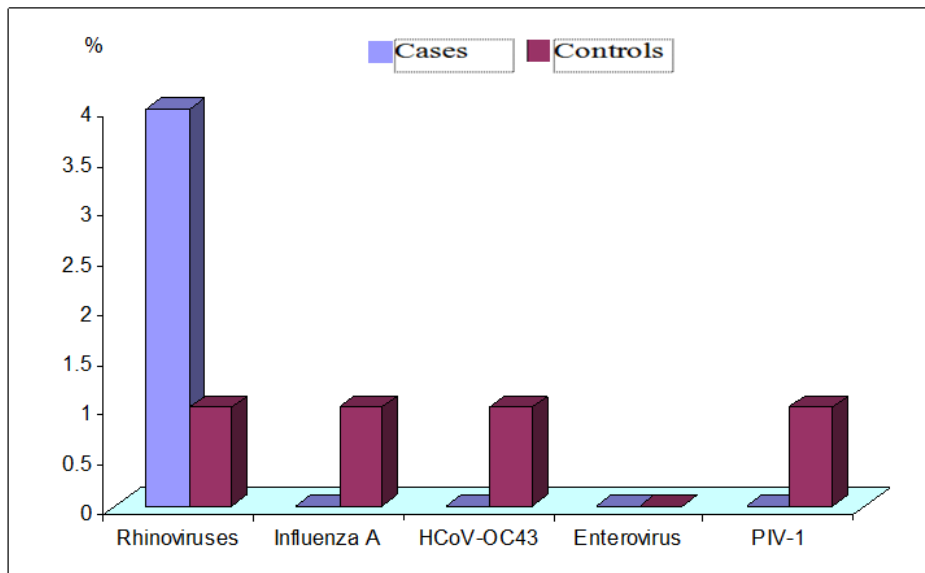


Fig. 2: Types of respiratory viruses from patients and controls by nasopharyngeal swabbing

DISCUSSION

Chronic rhinosinusitis (CRS) is a major public health problem with poor outcomes after standard medical treatment¹⁷. It was observed that the majority of exacerbations of CRS occur in seasons with high prevalence of respiratory viral infections that are incriminated in injury of nasal epithelial cells, and helping the access of bacteria into the nasal mucosa¹⁰. However, few studies have discussed the association between viral infections and CRS, so that the prevalence as well as the pathogenic impact of respiratory viruses on patients with CRS remains unknown¹³.

The main concern of this study was to assess the prevalence and types of respiratory viruses in nasal

mucosa of CRS patients to provide additional understanding of this association. This study was carried out on 30 CRS patients and 30 healthy controls matched for age and sex.

We found that the majority of our CRS patients were females (70%), while only 30% were male patients, yet with no statistically significant difference. This finding was in agreement with Chen and his coworkers who found that female patients were twice as likely as male patients. This could be explained by the smaller ostia in women that make them more susceptible to obstruction and subsequent infection, also there is an evidence that women are more likely to develop asthma and COPD¹⁸.

Concerning the associated co-morbidities in CRS patients, we found that the most common illness

associated with CRS in our patients was allergic rhinitis followed by asthma. Several reports supported the coexistence of asthma along with AR with faster occurrence of CRS^{19,20}. The association between CRS & AR was also described by Kennedy and Borish who found that the direct aeroallergen reaction might be the cause of this association²¹.

On performing the intradermal skin test, smoke was the most commonly detected allergen, followed by pollens, house dust mites, hay dust, mixed fungus, cotton and wool. Georgalas and his colleagues referred to similar findings in CRS patients especially in patients with nasal polyposis²².

A multiplex nested PCR was performed on nasal wash samples from our patients and control volunteers and a panel of respiratory viruses was detected from 53.3% of our CRS patients with no symptoms or signs of acute respiratory tract infection, while only 16.7% of the control group samples were positive for the studied viruses, with a statistically significant difference. This finding was met by results of Cho and coworkers who found respiratory viruses in 50.5% of their CRS patients¹³. Bousquet et al. postulated that respiratory viruses could stimulate chronic inflammation or cause acute exacerbation of CRS¹⁷. Moreover, Stephenson & his colleagues suggested that community-acquired respiratory viral infections may contribute in aggravating inflammation of CRS similar to bacteria and fungi²³. On the other hand, other researchers could not detect any respiratory viruses among their CRS patients and they concluded that persistence of respiratory viruses within the sinonasal mucosa is not proved to be a cause of ongoing inflammation in CRS^{11,24}.

To identify the possible relationship between prevalence of respiratory viruses among CRS patients and associated allergic conditions, we observed that 57.9% of CRS patients with AR had viral positive samples. This result was met by Liao et al. who detected respiratory viruses in adult CRS patients with AR, yet with no statistically significant differences regarding symptom scores or the presence of polyps¹². Respiratory viruses were isolated from 50% of nasal wash samples of CRS patients with asthma. This association was supported by Bachert and colleagues who demonstrated that a relation between asthma and CRS was found particularly in those with nasal polyps²⁵. Whereas, other researchers found that a high proportion of asthmatic patients had respiratory viruses in their respiratory secretions especially rhinoviruses, which were associated with asthma exacerbations²⁶.

In the present work, we found rhinovirus to be the most commonly detected virus from nasal wash samples among patients & controls. This finding was in accordance with other studies, postulating that RV was the most prevalent and major virus that had a significantly different detection rate in CRS patients and

control subjects¹². In addition, DeMuri & coworkers who also performed multiplex PCR for respiratory viruses, found that RV was most common virus²⁷. RV induces persistent changes in the local cytokine milieu and increases the bacterial adhesion to nasal epithelial cells, maintaining the effect of transient infection and contributes to disease exacerbations^{28,29}.

We compared results of nasal lavage & nasopharyngeal swabbing combined with oropharyngeal swabbing where the nasopharyngeal swab method detected respiratory viruses in 70% of cases and 50% of controls, while nasal wash samples revealed viruses in only 30% and 10% of the same patients' controls. This multitechnique approach improved the rate of viral detection and helped us to assess the prevalence of viruses among CRS patients included in the study. This was in agreement with Hammitt et al. who postulated that collection of combined nasal and oropharyngeal samples could potentially increase the rate of virus detection³⁰. However, Campbell et al. stated that the small increase in detection must be balanced by the cost of additional swabs, patient discomfort and non-compliance problems³¹.

Testing nasal wash samples detected only the presence of a single virus among positive samples, while nasopharyngeal swabs revealed the presence of not only single but sometimes multiple viruses in 30% of patients' & 10% of control positive samples. Heikkinen and his colleagues also referred to the importance of performing more than one sampling method to increase yield of viruses in nasopharyngeal secretions³². On the other hand, Mahdavinia et al. thought that nasal lavage would provide a diluted sample containing microbes from almost all areas in the sinonasal cavity and potentially pharyngeal area, and could be contaminated by skin microbes³³. Unfortunately collecting a large number of cases by the nasopharyngeal swab along with healthy controls is so difficult and would limit data to surgical patients with more severe disease.

In the current work, we performed the multiplex nested PCR assays with fast cyclic conditions for rapid amplification and detection of a wide range of respiratory viruses. This was recommended by Lam & his coworkers who stated that the sensitivity of multiplex nested PCR assays to detect viruses was 100- to 1,000-fold more sensitive than virus isolation by cell culture with overall positive rate as determined by multiplex nested PCR which was significantly higher than those of virus isolation and IFA¹⁶.

A panel of respiratory viruses was detected in our study, RV was the most commonly detected respiratory virus in 75% of nasal wash samples and 57 % of the nasopharyngeal swabbings, followed by Influenza A virus, HCoV-OC43, Enterovirus and Parainfluenza virus. Whereas, Cho et al. isolated rhinovirus, parainfluenza, influenza, RSV, HCoV-OC43,

adenovirus, enterovirus, and HMPV from samples of CRS patients using multiplex real-time PCR¹³. In comparison, we investigated nearly the same range of viruses with lowering the cost of reaction compared to multiplex real-time PCR, moreover, the nested PCR increased the specificity of the reaction.

CONCLUSIONS

The present study emphasizes the prevalence of respiratory viruses, especially RV, in nasal samples of CRS patients pointing to their role in the pathogenesis of the disease. The co-presence of CRS with AR and/or asthma in a percentage of our patients, is worth attention and suggests their relationship to disease progression. The multiplex nested PCR technique facilitated rapid and sensitive detection of the viruses.

Recommendations

We recommend further studies to use larger sample size together with sequencing of RNA of respiratory viruses for better knowledge of prevalent viruses in CRS.

Conflicts of interest

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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